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Application
for
United States Letters Patent

To all whom it may concern:

Be it known that **Hung-Teh Kao, Paul R. Hartig, and Theresa Branchek**

have invented certain new and useful improvements in

DNA ENCODING A HUMAN SEROTONIN ($5HT_2$) RECEPTOR AND USES THEREOF

of which the following is a full, clear and exact description.

DNA ENCODING A HUMAN SEROTONIN (5-HT_2) RECEPTOR AND USES THEREOF

Background of the Invention

Throughout this application various publications are referenced by full citations within parentheses. The disclosures of these publications in their entireties are hereby incorporated by reference in this application in order to more fully describe the state of the art to which this invention pertains.

Pharmacological studies, and more recently gene cloning, have established that multiple receptor subtypes exist for most, if not all, neurotransmitters. The existence of multiple receptor subtypes provides one mechanism by which a single neurotransmitter can elicit distinct cellular responses.

The variation in cellular response can be achieved by the association of individual receptor subtypes with different G proteins and different signalling systems. Further flexibility is provided by the ability of distinct receptors for the same ligand to activate or inhibit the same second messenger system.

Individual receptor subtypes reveal characteristic differences in their abilities to bind a number of ligands, but the structural basis for the distinct ligand-binding properties is not known. Physiologists and pharmacologists have attempted to specify particular biological functions or anatomical locations for some receptor subtypes, but this has met with limited success. Similarly, the biochemical mechanisms by which these receptors transduce signals across the cell surface have been difficult to ascertain without

having well-defined cell populations which express exclusively one receptor subtype.

Receptors for serotonin (5-hydroxytryptamine) are termed serotonin or 5-HT receptors. The 5-HT_2 receptor belongs to the family of rhodopsin-like signal transducers which are distinguished by their seven-transmembrane configuration and their functional linkage to G-proteins. While all the receptors of the serotonin type are recognized by serotonin, they are pharmacologically distinct and are encoded by separate genes. These receptors, known as subtypes, are generally coupled to different second messenger pathways that are linked through guanine-nucleotide regulatory (G) proteins. Among the serotonin receptors, 5-$HT1_A$, 5-$HT1_B$, and 5-$HT1_D$ receptors inhibit adenylate cyclase, and 5-$HT1_C$ and 5-HT_2 receptors activate phospholipase C pathways, stimulating breakdown of polyphosphoinositides.

Radioligand filtration binding techniques have been employed to characterize the serotonin receptor family (Schmidt and Peroutka, FASEB J. 3:2242 (1989)). Using these methods, at least two classes of G-protein coupled serotonin receptors have been described, 5-HT_1, and 5-HT_2. These differ in their selectivity for drugs. 5-HT_1 receptors display high (nanomolar) affinity for serotonin and can be labeled with [^{3}H] 5-HT. 5-HT_2 receptors display low affinity for serotonin but have high (nanomolar) affinity for antagonists such as Ketanserin, Mesulergine, Metergoline and d-LSD. Genes for the 5-HT_{1A} receptor (Fargin, et al., Nature 335:358-360 (1988); Kobilka, et al., Nature 329:75-79 (1987)) and the 5-HT_{1C} receptor (Julius, et al., Science 241:558-564 (1988)) have been isolated.

Applicants have cloned a human 5-HT_2 receptor, clone 6B, which has been transfected into a heterologous expression system, producing a membrane protein with binding properties

consistent with its preliminary characterization based on amino acid homology as the 5-HT_2 receptor subtype. The results from binding studies are consistent with the projected subtype based on amino acid sequence homology.

The receptor encoded by clone 6B shares numerous sequence and structural properties with the family of receptor molecules that has been predicted to span the lipid bilayer seven times. This family includes rhodopsin and related opsins (Nathans, J. and Hogness, D.S., Cell 34:807 (1983)), the α and ß adrenergic receptors (Dohlman, H.G., et al., Biochemistry 26:2657 (1987)), the muscarinic cholinergic receptors (Bonner, T.I., et al., Science 237:527 (1987)), the substance K neuropeptide receptor, (Masu, Y., et al., Nature 329:836 (1987)), the yeast mating factor receptors, (Burkholder, A.C. and Hartwell, L.H., Nucl. Acids Res. 13:8463(1985); Hagan, D.C., et al., Proc. Natl. Acad. Sci. USA 83:1418 (1986)); Nakayama, N. et al., EMBO J. 4:2643 (1985)), the serotonin receptor, and the oncogene c-mas, (Young, et al., Cell 45:711 (1986)). Each of these receptors is thought to transduce extracellular signals by interaction with guanine nucleotide-binding (G) proteins (Dohlman, H.G., et al., Biochemistry 26:2657 (1987); Dohlman, H.G., et al., Biochemistry 27:1813 (1988); O'Dowd, B.F., et al., Ann.Rev. Neurosci., in press).

Membranes of cells transfected with clone 6B bind both ^{3}H-Ketanserin and ^{3}H-DOB, demonstrating that the reported "hallucinogen receptor" must be an affinity state of the 5-HT_2 receptor rather than a distinct receptor subtype. Thus, the argument of Tietler (Lyon, et al. Mol. Pharm. 31: 194-199 (1987)) for multiple affinity states of the 5-HT_2 receptor is supported and that of Peroutka (Pierce, P.A., and S.J. Peroutka J. Neurochem. 52: 656-658 (1989)) for multiple 5-HT_2 receptor subtypes is not. This observation provides the opportunity to use the transfected human 5-

HT_2 receptor as a tool for the development of drugs which induce or which interfere with hallucinogenesis, caused either by disease processes or by drugs of abuse.

Strader, Sigal and Dixon recently published a model for the neurotransmitter binding site of G protein-coupled receptors (FASEB J. 3: 1825-1832 (1989)). According to this model, adrenergic receptors contain two serine residues in transmembrane segment V (TM5) which hydrogen bond to the catechol ring hydroxyl groups of adrenergic agonists. Serotonergic receptors, which must bind agonist ligands containing a single ring hydroxyl group, are distinguished by a the presence of a single serine residue in this region of TM5. The rat serotonin 5-HT_2 receptor sequence (Pritchett, et al., EMBO J. 13: 4135-4140 (1988)) shows a single serine residue in this region of TM5, as expected. Surprisingly, the human 5-HT_2 receptor sequence shown in Figure 2 violates this model by exhibiting two serine residues in this region, as would be expected for an adrenergic receptor. This raises the interesting possibility that the human 5-HT_2 receptor may have evolved the possibility of interacting with epinephrine, norepinephrine and adrenergic antagonists, in addition to its known interactions with serotonergic drugs. This possible acquisition by the human 5-HT_2 receptor of a neurotransmitter cross-reactivity may have functional consequences in the normal or diseased human brain. We hypothesize that the human 5-HT_2 receptor may have evolved the capacity to interact with two separate neurotransmitter systems, the serotonergic and adrenergic systems. Since both systems are widely distributed in the brain and both may act in a neuromodulatory fashion to activate receptors far from the neurotransmitter release site, it is conceivable that the human 5-HT_2 receptor may be activated by a wide array of both serotonergic and adrenergic nerve terminals. In that case, classical adrenergic and

serotonergic brain or peripheral nervous system functions may be mediated in part by this single receptor site. Thus, it may be possible to modulate serotonergic functions by administration of adrenergic drugs and to modulate adrenergic functions by administration of serotonergic drugs. These possibilities are currently under investigation in a variety of adrenergic and serotonergic binding, second messenger and physiological response assays.

Another interesting feature of the human 5-HT_2 receptor is the presence of a leucine zipper motif in transmembrane segment I (Figure 3). This motif, consisting of four or more leucine residues repeated every seventh amino acid residue of an alpha-helix, has been implicated as the site of protein-protein interactions in dimerizing, or multisubunit proteins (McCormack et al. Nature 340: 103 (1989)). The presence of this motif in the human 5-HT_2 receptor suggests that this receptor may dimerize in the membrane or may interact with other unidentified proteins (or with G proteins) via the leucine zipper of transmembrane segment I. This may have significant implications for the function of the human 5-HT_2 receptor. In addition, it may be possible to design drugs which interfere with the leucine zipper region of the 5-HT_2 receptor, thus modulating the functional activity of this serotonergic response system.

Summary of the Invention

The present invention provides an isolated nucleic acid molecule encoding a human 5-HT_2 receptor.

This invention also provides an isolated protein which is a human 5-HT_2 receptor.

The invention also provides vectors comprising DNA molecules encoding a human 5-HT_2 receptor, for example a plasmid comprising the DNA encoding the 5-HT_2 receptor, designated clone 6B.

Additionally, the present invention provides vectors adapted for expression in bacterial, yeast, or mammalian cells which comprise a DNA molecule encoding the 5-HT_2 receptor and the regulatory elements necessary for expression of the DNA in the cell.

The present invention further provides the transfected Ltk$^-$ cell designated L-NGC-5HT_2 and deposited under ATCC Accession No. .

In addition, the invention provides a DNA probe useful for detecting nucleic acid encoding the 5-HT_2 receptor comprising a nucleic acid molecule of at least about 15 nucleotides having a sequence complementary to a sequence included within the sequence shown in Figure 2.

This invention also provides a method for determining whether a ligand which is not known to be capable of binding to the 5-HT_2 receptor can bind to the 5-HT_2 receptor.

This invention also concerns an antibody directed to the human 5-HT_2 receptor.

This invention additionally concerns a monoclonal antibody directed to an epitope of the 5-HT_2 receptor present on the surface of a cell and having an amino acid sequence included within the amino acid sequence shown in Figure 2.

This invention concerns a method for detecting the presence of 5-HT_2 receptor on the surface of a cell.

This invention also concerns a method of screening drugs to identify drugs which specifically interact with, and bind to, the 5-HT_2 receptor.

Brief Description of the Figures

Figure 1. Restriction Map of Clone.

The bold region represents the human 5-HT_2 coding sequence. Restriction sites are indicated.

Figure 2. Nucleotide Sequence and Deduced Amino Acid Sequence of the Human 5-HT_2 Receptor.

Numbers indicate nucleotide position. DNA sequence of cDNA clone 6B was determined by the chain termination method of Sanger, et al., on denatured double-stranded plasmid templates (Chen and Seeburg, DNA 4:165,1985) using Sequenase. Deduced amino acid sequence by translation of a long open reading frame is shown.

Figure 3. Comparison of the Sequence Between the Rat and Human 5-HT_2 Receptors.

Amino acid sequences (single letter code) are pictured as a protein with the putative transmembrane domains traversing the membrane seven times. Differences are indicated as filled in circles.

Figure 4. Saturation Curves for the Binding of ^{3}H-Ketanserin to Stably Transfected Ltk$^-$ Cells.

The lower curve represents non-specific binding as defined by 10^{-6}M Mianserin. The middle curve is the calculated specific binding. For this experiment, K_D =0.678nM; B_{max} =0.602 pmole/mg protein. This dissociation constant is consistent with the designation of 6B as a 5-HT_2 receptor.

Detailed Description of the Invention

This invention provides an isolated nucleic acid molecule encoding a human 5-HT_2 receptor, for example a DNA molecule or a cDNA molecule.

This invention provides DNA encoding a 5-HT_2 receptor, for example the genomic DNA or cDNA having a coding sequence substantially the same as the coding sequence shown in Figure 2.

This invention provides an isolated protein which is a human 5-HT_2 receptor. An example of such a protein has substantially the same amino acid sequence as the amino acid sequence shown in Figure 2. A means for obtaining isolated human 5-HT_2 receptor is expressing DNA encoding the receptor in a suitable host, such as a bacterial, yeast, or mammalian cell, using methods well known in the art, and recovering the 5-HT_2 receptor after it has been expressed in such a host, again using methods well known in the art.

This invention provides vectors comprising DNA encoding a human 5-HT_2 receptor, and DNA and cDNA having a coding sequence substantially the same as the coding sequence shown in Figure 2. Some examples are a plasmid, such as pUC18, or a virus, or a bacteriophage such as lambda bacteriophage.

One example of a plasmid comprising DNA having a coding sequence substantially the same as the coding sequence shown in Figure 2 is the plasmid designated clone 6B.

This invention further provides a plasmid adapted for expression in a bacterial, yeast, or mammalian cell which comprises DNA encoding the 5-HT_2 receptor, or DNA or cDNA having a coding sequence substantially the same as the

coding sequence shown in Figure 2, and the regulatory elements necessary to express such DNA in the bacterial, yeast, or mammalian cell. As regards the latter, those skilled in the art will readily appreciate that numerous plasmids may be constructed utilizing existing plasmids and adapted as appropriate to contain the regulatory elements necessary to express the DNA in the mammalian cell. Numerous mammalian cells may be used including, for example, the mouse fibroblast cell NIH3T3, CHO cells, HeLa cells, etc. One example of a plasmid adapted for the expression of a cDNA molecule having a coding sequence substantially the same as the coding sequence shown in Figure 2 is the plasmid designated pMO5-6B described more fully hereinafter and deposited with the American Type Culture Collection under ATCC Accession No. .

This deposit and the other deposit discussed herein were made pursuant to, and in satisfaction of, the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and were made with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852.

This invention provides expression plasmids used to transfect mammalian cells, for example Ltk$^-$ cells, comprising plasmids adapted for expression in mammalian cells which comprise DNA encoding a human 5-HT_2 receptor, or comprising DNA or cDNA having a coding sequence substantially the same as the coding sequence shown in Figure 2. In one presently preferred embodiment this invention provides an Ltk$^-$ cell transfected with the plasmid designated pMO5-6B. This cell line is designated L-NGC-5HT_2 and is deposited under ATCC Accession No. . DNA encoding the 5-HT_2 receptor may be otherwise introduced into mammalian cells, e.g., by microinjection, to obtain

mammalian cells which comprise DNA, e.g., cDNA or a plasmid, encoding the 5-HT_2 receptor.

This invention further provides a method for determining whether a ligand, such as a known or putative drug, which is not known to be capable of binding to the 5-HT_2 receptor, can bind to the 5-HT_2 receptor. This method comprises contacting a mammalian cell expressing 5-HT_2 receptor the with the ligand under conditions permitting binding of ligands known to bind to the 5-HT_2 receptor, detecting the presence of any of the ligand bound to the 5-HT_2 receptor and thereby determining whether the ligand binds to the 5-HT_2 receptor. An example of such a mammalian cell is a mammalian cell comprising a plasmid which comprises a DNA molecule encoding a human 5-HT_2 receptor, or DNA or cDNA molecules having coding sequences substantially the same as the coding sequence shown in Figure 2. Another example of such a mammalian cell is an Ltk$^-$ cell comprising a plasmid which comprises a DNA molecule encoding a human 5-HT_2 receptor, or DNA or cDNA molecules having coding sequences substantially the same as the coding sequence shown in Figure 2.

This invention still further provides a method of detecting the presence of mRNA coding for the 5-HT_2 receptor in a cell which comprises obtaining total mRNA from the cell, using well known methods, and contacting the mRNA so obtained with the cDNA having a coding sequence substantially the same as the coding sequence encoding the 5-HT_2 receptor shown in Figure 2, under hybridizing conditions, detecting the presence of mRNA hybridized to the cDNA, and thereby detecting the presence of mRNA coding for the 5-HT_2 receptor by the cell.

This invention also provides a DNA probe useful for detecting in a sample nucleic acid encoding the 5-HT_2

receptor. Such a probe comprises a nucleic acid molecule of at least about 15 nucleotides having a sequence complementary to a sequence included within the sequence shown in Figure 2. Such nucleic acid probe technology is well known to those skilled in the art who will readily appreciate that such probes may vary greatly in length and may be labeled with a detectable label, such as a radioisotope or fluorescent dye, to facilitate detection of the probe.

This invention provides an antibody directed to the human 5-HT_2 receptor. Such an antibody may be serum-derived or monoclonal and may be prepared using methods well known in the art. For example, cells such as SR3T3 cells or Ltk$^-$ cells may be used as immunogens to raise such an antibody. Alternatively, synthetic peptides may be prepared using commercially available machines and the amino acid sequence shown in Figure 2. As a still further alternative, DNA, such as a cDNA or a fragment thereof, may be cloned and expressed and the resulting polypeptide recovered and used as an immunogen. One example of such an antibody is a monoclonal antibody directed to an epitope of the 5-HT_2 receptor present on the surface of a cell and having an amino acid sequence substantially the same as any part of the amino acid sequence shown in Figure 2.

Still further this invention provides a method of detecting the presence of the 5-HT_2 receptor on the surface of a cell which comprises contacting the cell with a monoclonal or serum-based antibody directed to an exposed epitope on the 5-HT_2 receptor under conditions permitting binding of the antibody to the 5-HT_2 receptor, and detecting the presence of the antibody bound to the cell, and thereby the presence of the 5-HT_2 receptor on the surface of the cell. Such a method is useful in determining whether a given cell is

defective relative to the expression of 5-HT_2 receptor on the surface of the cell.

Finally, this invention provides a method of screening drugs to identify drugs which specifically interact with, and bind to, the 5-HT_2 receptor on the surface of a cell. This method comprises contacting a mammalian cell which is expressing 5-HT_2 receptor with a plurality of drugs, known or putative, determining those drugs which bind to the mammalian cell, and thereby identifying drugs which specifically interact with, and bind to, the 5-HT_2 receptor. An example of a mammalian cell is the mammalian cell comprising a plasmid which comprises a DNA molecule encoding a human 5-HT_2 receptor, or DNA or cDNA molecules having coding sequences substantially the same as the coding sequence shown in Figure 2.

Specifically this invention relates to the first isolation of a human cDNA clone encoding the 5-HT_2 receptor and expressing a serotonergic binding site in Ltk$^-$ cells by transfecting the cells with the construct pMO5-6B. A mammalian cell line expressing a human 5-HT_2 receptor at the cell surface has been constructed, as determined by pharmacologic methods, thus establishing the first well-defined, cultured cell line with which to study the human 5-HT_2 receptor.

The cDNA molecule of the subject invention, which encodes the human 5-HT_2 receptor, is useful for obtaining genomic DNA, cDNA or mRNA from human, mammalian or other animal sources, or to isolate related cDNA or genomic clones by the screening of cDNA or genomic libraries. The DNA molecule is obtained by insertion of the whole molecule or fragments thereof into suitable vectors, such as plasmids or bacteriophages, wherein it is replicated and harvested following insertion into suitable bacterial host cells,

using methods well known in the art. DNA or RNA fragments derived from the isolated DNA molecule are useful as probes for 'in situ' hybridization or to locate tissues which express this gene, or for other hybridization assays for the presence of the gene or its mRNA in various biological tissues. In addition, synthesized oligonucleotides complementary to the sequence of the DNA molecule are useful as probes for the 5-HT_2 receptor gene, for its associated mRNA, or for the isolation of related genes by homology screening of genomic or cDNA libraries, or by the use of amplification techniques such as the Polymerase Chain Reaction.

This invention is also useful to obtain the protein, or fragments of the protein, encoded by the isolated cDNA molecule encoding the human 5-HT_2 receptor. Transfecting suitable hosts, such as bacterial, yeast or mammalian cells, with the DNA molecule or fragments thereof in suitable expression vectors such as the plasmid pSVL, using methods well known in the art, yields expression of the 5-HT_2 receptor or fragments thereof for direct uses or for experimental study.

Response systems are obtained by coupling the 5-HT_2 receptor encoded by the isolated cDNA molecule to an appropriate second messenger response system. These second messenger response systems include, but are not limited to, such systems as phosphoinositide hydrolysis, adenylate cyclase, guanylate cyclase or ion channels. The response system is obtained by transfection of the isolated cDNA molecule into a suitable host cell containing the desired second messenger system. Such a host system is isolated from pre-existing cell lines, or is generated by inserting appropriate components of second messenger systems into existing cell lines. Such a transfection system provides a complete

response system for investigation or assay of the activity of the 5-HT_2 receptor encoded by the isolated cDNA molecule.

This invention is useful to determine whether a ligand, such as a known or putative drug, is capable of binding to and/or activating the 5-HT_2 receptor encoded by the isolated cDNA molecule. Transfection of the isolated cDNA molecule into the cell systems described above provides an assay system for the ability of ligands to bind to and/or to activate the receptor encoded by the isolated DNA molecule. Transfection systems, such as those described above, are useful as living cell cultures for competitive binding assays between known or candidate drugs and ligands which bind to the receptor and which are labeled by radioactive, spectroscopic or other reagents. Membrane preparations containing the receptor isolated from transfected cells are also useful for competitive binding assays. Functional assays of second messenger systems or their sequelae in a transfection system act as assays for binding affinity and efficacy in the the activation of receptor function. Such a transfection system constitutes a "drug discovery system", useful for the identification of natural or synthetic compounds with potential for drug development that can be further modified or used directly as therapeutic compounds to activate or inhibit the natural functions of the 5HT-$_2$ receptor encoded by the isolated cDNA molecule. The transfection system is also useful for determining the affinity and efficacy of known drugs at the human 5-HT_2 receptor site.

This invention is useful to isolate the genomic DNA encoding the 5-HT_2 receptor so that transcriptional regulatory elements from the 5' untranslated region of the isolated gene, and other stability, processing, transcription, translation, and tissue specificity-determining regions from the 3' and 5' untranslated regions of the isolated gene, are made available for further research and application.

This invention is also useful to generate antibodies directed against the 5-HT_2 receptor protein encoded by the isolated cDNA molecule. Expression of the protein encoded by the isolated cDNA molecule, in transfection systems such as those described above, provides protein or fragments of protein which are further useful to generate monoclonal or polyclonal antibodies against the isolated receptor, using methods well known in the art. These antibodies are useful to detect the presence of the receptor encoded by the isolated cDNA molecule, or to inhibit the function of the receptor encoded by the isolated cDNA molecule, in living animals, in humans, or in biological tissues or fluids isolated from animals or humans.

This invention identifies an individual receptor subtype protein and tests whether pharmacological compounds interact with it for use in therapeutic treatments. Pharmacological compounds which are directed against specific receptor subtypes will provide effective new therapies with minimal side effects.

In summary, this invention identifies for the first time a human 5-HT_2 receptor protein, its amino acid sequence, and its human gene. The information and experimental tools provided by this discovery will be useful to generate new therapeutic agents, and new therapeutic or diagnostic assays for this new receptor protein, its associated mRNA or its associated genomic DNA.

The invention will be better understood by reference to the Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention as described more fully in the claims which follow thereafter.

Experimental Details

Isolation of a rat 5-HT_{1C} receptor cDNA clone

The rat 5-HT_{1C} gene was isolated as a prerequisite to isolating a clone for the human 5-HT_2 gene. This gene was obtained by isolating clones from a rat choroid plexus cDNA library specifically primed with an oligomer complementary to the 3' untranslated region of the rat 5-HT_{1C} receptor. Oligomers complementary to the rat 5-HT_{1C} published sequence (Julius, et al., Science 241: 558-564 (1988) were labeled with ^{32}P-ATP and T4 Kinase (Maniatis et al, 1982). Clones showing positive hybridization to the probe were picked and inserts subcloned into the sequencing vector pUR250 (Ruther, Nucl. Acids Res 10:5765-5772 (1982)). Sequencing via the Sanger dideoxy method (Sanger, et al., Proc. Natl. Acad. Sci. 74:5463-5467 (1977)) confirmed the isolation of a clone containing the entire coding region of the gene.

Isolation of a human 5-HT_2 receptor cDNA clone.

Initially, a human brain stem cDNA library provided by Stratagene (La Jolla, California) was screened using a probe made from a rat 5-HT_{1C} receptor gene. This gene had been subcloned into the vector pGem1 (Sma I to Eco RI; Promega-Biotec; Madison, Wisconsin). Sense strand RNA transcribed by Sp6 polymerase served as a template for the generation of a high specific activity cDNA which was produced by reverse transcription with AMV reverse transcriptase and random primers. The reason for using this particular method was to avoid background problems we have encountered when generating probes using other methods.

Hybridization was performed at 60°C in a solution containing 5X SSC (1X SSC is 0.15 M sodium chloride, 0.015 M sodium citrate), 2X Denhardt's (0.02% polyvinyl-pyrrvolidone, 0.02% Ficoll, and 0.02% bovine serum albumin), 100 mM sodium

phosphate pH 8.0, 25 mM EDTA, 0.1% SDS (sodium dodecyl sulfate) and 100 μg/ml of sonicated salmon sperm DNA. The filters were washed at 60°C in 0.1X SSC containing 0.1% sodium dodecyl sulfate (SDS) and exposed at -70°C to Kodak XAR film in the presence of an intensifying screen. Lambda phage hybridizing to the probe were plaque purified and plasmid DNA (in the vector pBluescript) self-excised from the lambda vector (lambda ZAP).

One clone (Clone 15) displayed high homology to the published sequence of a rat 5-HT_2 receptor clone (Pritchett, et al., EMBO J. 7: 4135-4140 (1988)). However, this clone did not contain the entire coding region and was therefore nonfunctional.

Isolation of a functional human 5-HT_2 receptor CDNA clone

^{32}P-labeled oligomers complementary to the 5' end of Clone 15 were used to probe a human temporal cortex cDNA library (Stratagene, La Jolla, California). Methods for labeling and hybridization are identical to the above. Four independent clones were isolated with the probe, and one, designated 6B, was selected for further studies.

Clone 6B contained the entire coding region for a 5-HT_2 receptor and was therefore predicted to be functional. The sequence of this clone is displayed in Figure 2. Clone 6B was subcloned into the mammalian expression vector pMO5 to obtain the construct pMO5-6B.

DNA sequencing

Nucleotide sequence analysis was done by the Sanger dideoxy nucleotide chain-termination method (Sanger, et al., Proc. Natl. Acad. Sci., 74: 5463-5467, 1977) on denatured double-stranded plasmid templates (Chen and Seeburg, DNA 4: 165, 1985) using Sequenase (U.S. Biochemical Corp., Cleveland,Ohio).

^{3}H-Ketanserin(64.9/mmol;DuPont-NEN, Wilmington, DE) Binding to Transiently Transfected Cos-7 Cells:

^{3}H-Ketanserin was used as a radioligand to detect expression of the 5-HT_2 gene in transiently transfected Cos-7 cells. Membranes were incubated in 96 well microtiter plates at 37°C for 15' in solution containing buffer [50 mM Tris Cl, 0.5mM EDTA, 10mM $MgSO_4$, 0.1% ascorbate and 10 μM pargyline pH 7.6] 1-2 nM ^{3}H-Ketanserin (64-9/mmol; DuPont-NEN, Wilmington, DE), 20-40 μg/0.25 ml protein, and drugs. The total reaction volume was 0.25ml. The reaction was terminated by filtration through GF/B filters using a Brandel Cell Harvester Model 48R (Brandel, Gaithersburg, MD). Filters were washed 5 x 1 sec. with iced buffer to reduce non-specific binding. Dried filters were transferred to scintillation vials and counted by liquid scintillation spectroscopy using a Beckman LS 1701 LSC. Two and one half ml of Formula 963 (Beckman Instruments, Fullerton, CA) was used as cocktail. Specific binding was 95% of total binding in transiently transfected cells.

^{3}H-DOB (20.8 Ci/mmole; DuPont-NEN; Wilmington, DE) Binding to Transiently Transfected Cos-7 Cells.

Membranes were incubated identically as described for ^{3}H-Ketanserin (above). ^{3}H-DOB (20.8 Ci/mmol; DuPont-NEN; Wilmington, DE) was used as a radioligand at a final concentration of 1-2 nM. Specific binding was 70% of total binding in transiently transfected Cos-7 cells.

Saturation Studies:

To determine the equilibrium dissociation constant of the 5-HT_2 receptor, saturation analysis was performed using ^{3}H-Ketanserin as a radioligand. The concentration of the ^{3}H-Ketanserin covered a range between 0.25 and 20 nM. Mianserin at 1.0 μM was used to define specific binding. Incubation time was 60 minutes. All conditions were as described above. Data was analyzed by computer assisted non-linear

regression (Accufit Saturation; Lundon Software; Chagrin Falls, OH).

Method for Binding Assays On a Stable Cell Line:
Subsequent to analysis in transiently transfected cells, the 5-HT_2 clone was expressed as a stable transfectant in mouse Ltk$^-$ cells. Further characterization was done on these cells as described above. Specific binding was 75-85% of the total binding.

Competition studies for ^{3}H-Ketanserin binding were performed by adding increasing concentrations of test drug to the reaction. 10-12 different concentrations of each ligand were tested and spanned the expected IC_{50} range as determined from literature values. Data were analyzed by computer-assisted analysis (Accufit Competition; Lundon Software; Chagrin Falls, OH).

Experimental Results:

Nucleotide Sequence and Deduced Amino Acid Sequence of the Receptor Encoded by Clone 6B.
DNA sequence information obtained from clone 6B is shown in Figure 2. An open reading frame extending from an ATG codon at position 1 to a stop codon at position 1414 can encode a protein 471 amino acids in length, having a relative molecular mass (M_r) of 52,542. A comparison of this protein sequence with previously characterized neurotransmitter receptors indicates that clone 6B is a new member of a family of molecules which span the lipid bilayer seven times and couple to guanine nucleotide regulatory proteins (the G protein-coupled receptor family). A variety of structural features which are invariant in this family were present in clone 6B. The greatest homology was found between clone 6B and the rat 5-HT_{1C} and rat 5-HT_2 receptors. Overall, 90% sequence conservation between the rat and human 5-HT_2 clones

was observed over 471 amino acids. There is considerable divergence between rat and human protein sequences at the extracellular amino and cytoplasmic carboxy termino. Only 76% of the residues are conserved in these regions. In contrast, between these regions, which included transmembrane region domains and transmembrane loops, 98% of the residues are conserved.

Receptor Expression in Transfected Mammalian Cells

In order to confirm the functional identity of the newly isolated gene we have expressed clone 6B in cultured cell lines. A DNA fragment containing the entire coding region was subcloned into the expression vector pMO5. The resulting plasmid pMO5-6B was transiently introduced into Cos-7 cells using the DEAE-dextran protocol (Cullen, Methods in Enz. 152: 684-704, 1987).

Stable cell lines were produced by cotransfection with the plasmid containing the bacterial gene aminoglycoside phosphtransferase into Ltk$^-$ cells (American Type Culture Collection, Rockville, MD, Cell Line CCL 1,3) using the calcium phosphatase technique (Protocol & kit obtained from Specialty Media, Inc. Lavallette, NJ). Clones expressing aminoglycoside transferase were selected for the addition 1 mg/ml G418 (Gibco Laboratories, Grand Island, NY) to the culture medium. ^{3}H-Ketanserin and ^{3}H-DOB binding were used to monitor 5-HT$_2$ receptor gene expression in these clones. Three out of sixteen clones displayed specific binding of both ^{3}H- Ketanserin and ^{3}H-DOB. The remainder bound neither radioligand.

Cos-7 cells or Ltk$^-$ cells were pseudotransfected with pMO5 not containing an insert in order to assess endogenous levels of ligand binding. At 1nM or 2nM radioligand, no significant specific binding was detected. The background was low [100 CPM for ^{3}H-Ketanserin and 20 CPM for ^{3}H-DOB]

for both cell lines. Therefore, Cos-7 and Ltk$^-$ cells provide useful models for transfection of a putative 5-HT_2 receptor. Transiently transfected Cos-7 cells bound ^{3}H-Ketanserin with high affinity, and with an estimated site density of 0.95 -1.6 pm/mg protein. These cells also bound ^{3}H-DOB with high affinity and with a site density of 0.26 - 0.5 pm/mg protein. ^{3}H-DOB binding constituted approximately 30% of the ^{3}H- Ketanserin binding sites.

Full saturation analysis was performed on stable cell lines expressing the gene. Ltk$^-$ cells transfected with pMO5-6B bound ^{3}H-Ketanserin saturably, specifically, and with high affinity. The binding constants were evaluated by computer-assisted nonlinear regression using Accufit (Lundon Software, Chagrin Falls, OH). The equilibrium dissociation constant was 0.678 $\pm$ .13 nM and the B_{max} = 0.602 $\pm$ .07 pm/mg protein (See Figure 4). Further characterization was accomplished by performing competition experiments for a series of drugs. Analysis of the competition data was accomplished using the computer-assisted nonlinear regression program Accucomp. Data are shown in Table 1.

TABLE 1

PROPERTIES OF 3H-KETANSERIN BINDING TO THE
CLONED HUMAN 5-HT2 RECEPTOR EXPRESSED IN LTK- CELLS.

DRUG	K_i (nM) HUMAN	n	K_i (nM) HUMAN CORTEX	K_i (nM) RAT CORTEX[2]
SPIPERONE	0.22 ± .03	3	0.42[1]	1.5
5-HT	224.0 ± 22	4	174[2]	79
MESULERGINE	146 ± 5	2	158[2]	5
RITANSERIN	1.29	1	1.26[2]	7.2
CYPROHEPTADINE	2.95 ± 0.10	2	6.3[2]	1.8
METHYSERGIDE	2.62 ± 0.12	2	2.5[2]	4
BUTACLAMOL	9.0	1	2.4[1]	-
5-CT	3032	1	813[2]	19953

1. Lyon, et al., Mol. Pharmacol. 31: 194-199 (1987).

2. Hoyer, et al., Brain Res. 376: 97-107 (1986).

The high affinity of pMO5-6B transfected Cos-7 or Ltk$^-$ membranes for ^{3}H-Ketanserin indicates that this clone can code for the production of 5-HT$_2$ binding site in its otherwise naive host cells.

Discussion

Applicants have cloned and characterized a cDNA molecule encoding an human 5-HT$_2$ receptor. The expression of this cDNA clone in Cos-7 cells and Ltk$^-$ cells results in the appearance of this type of receptor on the cell surface.

Binding competition studies of pMO5-6B transfected Cos-7 or Ltk$^-$ cell membranes with ^{3}H-Ketanserin (Table 1) indicate that this clone is a 5-HT$_2$ receptor. Furthermore, the pharmacological profile of the cloned human receptor closely matches that of the ^{3}H-Ketanserin binding to the 5-HT$_2$ receptor in human cortical membranes rather than in rat cortical membranes even though the human receptor was expressed (by transfection) in a mouse fibroblast cell line (Ltk$^-$). This species difference is particularly evident for Mesulergine (see Table 1). This data strongly suggests that the species differences in pharmacological binding properties of the human 5-HT$_2$ receptor arise from amino acid differences in this protein rather than the cellular environment in which it is translated, processed and inserted in the cell membrane. Thus, the key parameter in obtaining a human-like pharmacology of the 5-HT$_2$ receptor appears to be the human gene sequence, rather than the cell type in which it is expressed. Therefore, isolation of the human gene provides the critical tool needed for the development of heterologous expression systems which will accurately model the pharmacological properties of human brain tissue. Moreover, the fact that these transfected cell membranes bind both ^{3}H-Ketanserin and ^{3}H-DOB demonstrates that the reported "hallucinogen receptor" must

be an affinity state of the 5-HT_2 receptor rather than a distinct receptor subtype. This observation provides the opportunity to use the transfected human 5-HT_2 receptor as a tool for the development of drugs which induce or which interfere with hallucinogenesis, caused either by disease processes or by drugs of abuse.